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<b>(54) Title:</b> <i>IN UTERO</i> TRANSPLANTATION OF HUMAN MESENCHYMAL STEM CELLS  <b>(57) Abstract</b>  The present invention is directed to mesenchymal stem cells for transplantation <i>in utero</i> . Such mesenchymal stem cells may be employed in a method of treating a fetus, or in a method of engrafting mesenchymal stem cells, or in a method of preparing an organ for transplantation.		

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**IN UTERO TRANSPLANTATION OF  
HUMAN MESENCHYMAL STEM CELLS**

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This application claims priority based on provisional application Serial No. 60/108,357, filed November 13, 1998.

The present invention relates to the field of cell therapy, and more particularly to the field of in vivo gene therapy by administering mesenchymal stem cells.

**Background of the Invention**

Certain diseases, including inherited metabolic diseases, may produce irreversible damage to the fetus before birth.

In utero hematopoietic stem cell transplantation is a potential therapy for a large number of immunodeficiency diseases, hemoglobinopathies and others. Advantages for the fetal recipient include potential immunologic tolerance for transplanted cells, and rapid expansion of the hematopoietic compartment with formation of new "niches" for the competitive engraftment of donor cells. The existence of natural models of hematopoietic chimerism in dizygotic twins which share cross placental circulation during development supports the potential to achieve high levels of donor cell chimerism with prenatal transplantation in recipients with normal hematopoiesis.

Experimental work in sheep and other animal models has successfully achieved long-term multilineage allogeneic or xenogeneic hematopoietic chimerism in immunopermissive fetuses without the requirement for myeloablation or immunosuppression. This chimerism in immuno permissive fetuses has been shown to be secondary to the engraftment of true pluripotent hematopoietic stem cells. Limited clinical success has been achieved in immunodeficiency disorders in which there is a selective advantage for donor cells. However in most diseases this selective advantage does not exist and engraftment has been absent or low. Limited or lack of engraftment, both in terms of cell numbers and in terms of differentiated cell types, currently represents an obstacle to expanded clinical application of in utero stem cell transplantation. The requirement for transplantation during the brief period of immunopermissiveness is another obstacle of the establishment of a successful therapy.

Mesenchymal stem cells are the formative pluripotential cells found *inter alia* in bone marrow, blood, dermis and periosteum that are capable of differentiation into any of the mesenchymal or connective tissues, for example, bone, cartilage, muscle, stroma, tendon, and fat.

This homogeneous population of cells can be passaged in culture and may be characterized by the lack of hematopoietic cell markers and by the presence of a unique set of surface antigens. Under specific conditions they have been induced to form bone, cartilage, adipose tissue, tendon, and muscle, and in their undifferentiated state, resemble roughly stromal fibroblasts and can support hematopoiesis as evidenced by the support of LT-CIC in long term bone marrow culture. Preliminary in vivo studies suggest that these cells home to bone marrow of post-natal recipients after intravenous administration and can accelerate constitution after myeloablative conditioning regimes.

Therefore it is an object of the invention to increase donor cell engraftment in fetal recipients. Another object is to regenerate damaged or diseased tissue by providing to a fetal recipient donor cells which can differentiate in situ. Yet another object is to prepare chimeric organs and tissues. Still another object is to treat a

recipient by administering mesenchymal stem cells which has been modified to express a therapeutic gene product.

### Summary of the Invention

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It has been discovered that mesenchymal stem cells (MSCs) transplanted into a fetus in utero then were distributed throughout the fetus. The MSCs remained viable and differentiated into cellular types appropriate for the tissue or organ in which they engrafted. Surprisingly, the MSCs and their differentiated progeny were not rejected by immunocompetent hosts. The MSCs can be used for cellular therapy and tissue engineering.

Normal MSCs express and secrete a number of cytokines, including G-CSF, SCF, LIF, M-CSF, IL-6, and IL-11. (Haynesworth, et al., *J. Cell Physiol.*, Vol. 166, No. 3, pgs. 585-592 (March 1996)). As such, when functional non-self MSCs are transplanted into a fetus having defective autologous MSCs, "gene therapy" thereby is performed on the host by virtue of the delivery of said functional MSCs.

It also has been discovered that when mesenchymal stem cells, which have been modified to carry exogenous genetic material of interest, are induced to differentiate, the progeny cells also carry the new genetic material. These transduced cells are able to express the exogenous gene product. Thus transduced mesenchymal stem cells and the cells differentiated therefrom can be used for applications where treatment using such modified cells is beneficial. For example, these modified cells can be used as a delivery system for therapeutic proteins encoded by the exogenous gene for treatment of inherited and/or acquired disorders of blood coagulation and wound healing.

Accordingly, the present invention provides a method of obtaining genetically modified mesenchymal stem cells, comprising transducing mesenchymal stem cells with exogenous genetic material and placing the transduced cells under conditions suitable for differentiation of the mesenchymal stem cells into lineages which then contain the exogenous genetic material.

Accordingly, the present invention is directed to a method for effecting gene therapy in vivo by administering human mesenchymal stem cells in utero. The

mesenchymal stem cells can differentiate into specific cell lineages depending on the environment and integrate with like tissue to effect repair of tissue defects.

5 In another embodiment of this aspect, the mesenchymal stem cells can be transduced with exogenous genetic material such that a gene product will be expressed by the mesenchymal stem cell or its differentiated progeny in vivo to provide a desired therapeutic effect.

10 In another aspect, the present invention involves a method for treating a subject in need thereof by administering human mesenchymal stem cells in an amount effective to enhance the in vivo distribution and engraftment of mesenchymal stem cells.

15 In another embodiment of this aspect, the mesenchymal stem cells can be transduced with exogenous genetic material such that a gene product will be expressed by the mesenchymal stem cell or its differentiated progeny in vivo to provide a desired therapeutic effect.

#### Brief Description of the Drawings

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Figure 1 is a photograph of liver tissue at 9 weeks after mesenchymal stem cells were injected intraperitoneally at 65 days gestation.

25 Figure 2 is a photograph of bone marrow at 9 weeks after mesenchymal stem cells were injected intraperitoneally at 85 days gestation.

Figure 3 is a photograph of heart tissue at 9 weeks after mesenchymal stem cells were injected intraperitoneally at 65 days gestation.

30 Figure 4 is a photograph of thymus tissue at 9 weeks after mesenchymal stem cells were injected intraperitoneally at 85 days gestation.

Figure 5 shows results of PCR analysis for human-specific  $\beta$ -2 microglobulin performed on tissue samples of liver (Lv), spleen (Sp), bone marrow (BM), thymus (Th), lung (Lg), brain (Br), and blood (Bd).

5        Figure 6A is a photograph of a gel showing the presence of human  $\beta$ -2 microglobulin DNA in liver, spleen, lung, bone marrow, thymus, brain, and blood isolated from sheep fetuses at 65 or 85 days gestation, wherein the sheep fetuses were given human mesenchymal stem cells in utero. Figures 6B, 6C, 6D, 6E, and 6F are photographs of slides showing the presence of human cells in sheep fetal  
10    liver, spleen, bone marrow, thymus, and lung, respectively.

Figure 7A is a photograph of a slide showing human mesenchymal stem cells, in the cardiac muscle of sheep fetuses stained with anti-human  $\beta$ -2 microglobulin. Figures 7B and 7C are photographs of slides showing human cells in  
15    the cardiac muscle of sheep fetuses stained with anti-human  $\beta$ -2 microglobulin and SERCA-2.

Figures 8A and 8B are photographs of slides showing the presence of human  $\beta$ -2 microglobulin through nickel chloride staining in cartilage lacunae of lambs  
20    given human mesenchymal stem cells in utero at 65 days gestation, and wherein the cartilage was harvested at 2 months and 5 months after transplantation, respectively.

Figure 9 shows photographs of slides of human cells, contacted with human-specific anti-CD23 antibody, found in the bone marrow of sheep at 5 months after in  
25    utero transplantation of human mesenchymal stem cells. Figure 9A is a control showing normal ovine tissue contacted with human-specific anti-CD23 antibody. Figures 9B through 9D show CD23+ human cells in ovine bone marrow at increasing magnification.

30        Figure 10 shows photographs of slides of human cells, contacted with human-specific anti-CD74 antibody, found in the bone marrow of sheep at 5 months after in utero transplantation of human mesenchymal stem cells. Figure 10A is a control showing normal ovine tissue contacted with human-specific anti-CD74

antibody. Figures 10B through 10D show CD74+ human cells in ovine thymus at increasing magnification.

Figures 11A and 11B are photographs of slides showing human  $\beta$ -2  
5 microglobulin positive cells in the central nervous system of sheep at 5 months after the sheep were given mesenchymal stem cells in utero.

### Detailed Description of the Invention

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The human/sheep model is a unique model of widely disparate xenogeneic chimerism in which human hematopoietic stem cells survive for years in the sheep bone marrow after in utero transplantation. These cells can establish multilineage long term engraftment after retransplantation in utero into second generation  
15 recipients proving the engraftment of pluripotent hematopoietic stem cells. The system is limited by species specificity of hematopoietic cytokines. The sheep microenvironment can support the viability of human HSCs and progenitors but human cytokine administration is required to drive the cells toward differentiation and peripheral expression. Human cells can be detected in this system using a  
20 variety of sensitive methodologies including flow cytometry, fluorescence in situ hybridization, immunohistochemistry, and PCR.

The present invention relates generally to the use of human mesenchymal stem cells and to compositions comprising human mesenchymal stem cells for in  
25 utero administration.

In order to obtain subject human mesenchymal stem cells for the methods described herein, mesenchymal stem cells can be recovered from other cells in the bone marrow or other mesenchymal stem cell source. (Pittenger, supra.) Bone marrow  
30 cells may be obtained from iliac crest, femora, tibiae, spine, rib or other medullary spaces. Other sources of human mesenchymal stem cells include embryonic yolk sac, placenta, umbilical cord, fetal and adolescent skin, and blood. The presence of mesenchymal stem cells in the culture colonies may be verified by specific cell surface markers which are identified with unique monoclonal antibodies, see, e.g.,



U.S. Patent No. 5,486,359. These isolated mesenchymal cell populations display epitopic characteristics associated only with mesenchymal stem cells, have the ability to regenerate in culture without differentiating, and have the ability to differentiate into specific mesenchymal lineages *in vitro* or *in vivo*.

5

The mesenchymal stem cell populations can be autologous, allogeneic, or xenogeneic to the recipient. Preferably, mesenchymal stem cells are from the same species as the recipient. Most preferably, the MSCs are human in origin.

10       Accordingly, any process that is useful to recover mesenchymal stem cells from human tissue may be utilized to result in a population of cells comprising mostly mesenchymal stem cells. In one aspect, a method of isolating mesenchymal stem cells comprises the steps of providing a tissue specimen containing mesenchymal stem cells, preferably bone marrow; isolating the mesenchymal stem  
15       cells from the specimen, for example by density gradient centrifugation; adding the isolated cells to a medium which contains factors that stimulate mesenchymal stem cell growth without differentiation and allows, when cultured, for the selective adherence of only the mesenchymal stem cells to a substrate surface; culturing the specimen-medium mixture; and removing the non-adherent matter from the  
20       substrate surface.

      According to the method of the present invention, the isolated mesenchymal stem cells are culture expanded in appropriate media, i.e. cultured by methods which favor cell growth of the enriched cell populations. In general, the cells are plated at  
25       a density of  $0.05\text{--}2 \times 10^5$  cells/cm<sup>2</sup>, preferably at a density of  $0.5\text{--}10 \times 10^4$  cells/cm<sup>2</sup>.

      The cells may be characterized prior to, during, and after culture to determine the composition of the cell population, for example by flow cytometric analysis (FACS). The human mesenchymal stem cells can be stained with human  
30       mesenchymal stem cell-specific monoclonal antibodies.

      The culture conditions such as temperature, pH, and the like, are those previously used with the cells utilized in this invention and will be apparent to one of skill in the art.

The mesenchymal stem cells produced according to the methods described herein can be used to provide a reliable and constant source of mesenchymal stem cells for individuals in need thereof, e.g. those in need of cellular therapy, tissue engineering or regeneration and gene therapy.

Another aspect of the present invention relates to the introduction of genes into the mesenchymal stem cells such that the mesenchymal stem cells and progeny of the cells carry the new genetic material.

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Thus, in accordance with this aspect of the invention, the mesenchymal stem cells can be modified with genetic material of interest. The mesenchymal stem cells are able to express the product of the gene expression and, with a signal sequence, secrete the expression product. These modified cells can then be administered to a target, i.e., in need of mesenchymal stem cells or the gene expression product, where the expressed product will have a beneficial effect.

Thus, genes can be introduced into cells which are then returned to the autologous donor or provided to a non-self recipient where the expression of the gene will have a therapeutic effect. For example, mesenchymal stem cells may be genetically engineered to express therapeutic proteins. Those that may be mentioned include providing continuous delivery of insulin, which at present must be isolated from the pancreas and extensively purified or manufactured in vitro recombinantly and then injected into the body by those whose insulin production or utilization is impaired. Genetically engineered human mesenchymal stem cells can also be used for the production of clotting factors. Persons suffering from hemophilia A lack a protein called Factor VIII, which is involved in clotting. A recombinant Factor VIII product is now available and is administered by injection (Kogenate®, Bayer, Berkeley, CA). Incorporation of genetic material of interest into human stem cells and other types of cells is particularly valuable in the treatment of inherited and acquired disease. Inherited disorders that could be treated in this way include disorders of amino acid metabolism, such as Fabry's Disease, Gaucher's Disease, histidinuria or familial hypocholesterolemia; and disorders of nucleic acid metabolism, such as hereditary orotic aciduria. Human stem cells transduced with a

gene encoding the missing or inadequately produced substance can be used to produce it in sufficient quantities. For example, a Fabry's patient incapable of producing sufficient quantities of the enzyme alpha galactosidase A could be given MSCs transduced with the gene coding for that enzyme.

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Some genetic diseases cause damage to the fetus or early post-natal individual; accordingly it is desirable to deliver the needed gene product in utero. Mesenchymal stem cells are advantageous particularly for gene therapy because they may be transduced with high efficiency, are long-lived and retain the ability to produce large numbers of daughter cells. Transduced MSCs express exogenous genes at high levels for long periods. This expression can continue through and after terminal differentiation. (Allay, et al., Human Gene Therapy, Vol. 8, No. 12, pgs. 1417-1427 (August 10, 1997).)

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The mesenchymal stem cells may be genetically modified by incorporation of genetic material into the cells, for example using recombinant expression vectors.

As used herein "recombinant expression vector" refers to a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

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The mesenchymal stem cells thus may have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit as a component of a resident plasmid. Cells may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, for example. Cells

may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide.

5 Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is MGIN,  
10 derived from murine embryonic stem cells. Generally regarding retroviral mediated gene transfer, see McLachlin et al.(1990).

A preferred retroviral packaging cell line is described in U.S. Patent No. 5,910,434, the contents of which are incorporated herein by reference. Such cell line  
15 permits very high levels of transfection, *i.e.*, greater than 80%.

The nucleic acid sequence encoding the polypeptide is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, TRAP promoter, adenoviral promoters, such as the adenoviral major late  
20 promoter; the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoA1 promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral long terminal repeats (LTRs);  
25 ITRs; the  $\beta$ -actin promoter; and human growth hormone promoters; the GPIIb promoter. The promoter also may be the native promoter that controls the gene encoding the polypeptide. These vectors also make it possible to regulate the production of the polypeptide by the engineered progenitor cells. The selection of a suitable promoter will be apparent to those skilled in the art. The retroviral LTR is  
30 preferred.

It is also possible to use vehicles other than retroviruses to genetically engineer or modify the mesenchymal stem cells. Genetic information of interest can

be introduced by means of any virus which can express the new genetic material in such cells. For example, SV40, herpes virus, adenovirus, adeno-associated virus, and human papillomavirus can be used for this purpose.

- 5           In addition, the expression vectors may contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed cells such as dihydrofolate reductase, neomycin resistance or green fluorescent protein (GFP).

10           The mesenchymal stem cells may be transfected through other means known in the art. Such means include, but are not limited to transfection mediated by calcium phosphate or DEAE-dextran; transfection mediated by the polycation Polybrene (Kawai and Nishizawa 1984; Chaney et al. 1986); protoplast fusion (Robert de Saint Vincent et al. 1981; Schaffner 1980; Rassoulzadegan et al. 1982); electroporation (Neumann et al. 1982; Zimmermann 1982; Boggs et al. 1986);  
15           liposomes (see, e.g. Mannino and Gould-Fogerite (1988)), either through encapsulation of DNA or RNA within liposomes, followed by fusion of the liposomes with the cell membrane or, DNA coated with a synthetic cationic lipid can be introduced into cells by fusion (Feigner et al. (1987); Feigner and Holm 1989; Maurer 1989).

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          The present invention further makes it possible to genetically engineer mesenchymal stem cells in such a manner that they produce, *in vitro* or *in vivo* polypeptides, hormones and proteins not normally produced in human mesenchymal stem cells in biologically significant amounts or produced in small amounts but in  
25           situations in which increased expression would lead to a therapeutic benefit. These products then would be secreted into the surrounding media or purified from the cells. The human mesenchymal stem cells formed in this way can serve as continuous short-term or long-term production systems of the expressed substance. Alternatively, the cells could be modified such that a protein normally expressed  
30           will be expressed at much lower levels. This can be accomplished with antisense nucleic acid technology, catalytic enzyme expression, single chain antibody expression, and the like.

This technology may be used to produce additional copies of essential genes to allow augmented expression by the mesenchymal stem cells of certain gene products *in vivo*. These genes can be, for example, hormones, matrix proteins, cell membrane proteins, cytokines, adhesion molecules, "rebuilding" proteins important in tissue repair. The expression of the exogenous genetic material *in vivo*, is often referred to as "gene therapy". Disease states and procedures for which such treatments have application include genetic disorders and diseases of blood and the immune system. Cell delivery of the transformed cells may be effected using various methods and includes intravenous or intraperitoneal infusion and direct depot injection into periosteal, bone marrow and subcutaneous sites.

The mesenchymal stem cells of the present invention may be administered to the fetus using methods generally known in the art.

In another aspect, the present invention provides a method of modifying fetal organs of a first species by administering mesenchymal stem cells of a second species to a fetus of the first species in utero. In a preferred embodiment, human mesenchymal stem cells are administered to a non-human fetus in utero. Although the scope of this aspect of the present invention is not to be limited to any theoretical reasoning, it is believed that the non-human fetal organs may be less immunogenic, in the context of subsequent transplants into humans than unmodified organs. (See, for example, published PCT Application No. WO99/47163.) Thus, the modified non-human organs may be more suitable for transplantation.

This aspect of the present invention is applicable particularly to the transplantation of animal organs into humans. Such organs include, but are not limited to, the heart, pancreas, kidney, skin, liver, thymus, spleen, bone marrow, cartilage, and bone. For example, human mesenchymal stem cells are administered to a non-human animal fetus. The MSCs preferably are autologous to the human patient. After birth, the non-human animal is raised to an appropriate age and/or size such that the size of the organ or organs to be transplanted approximates the size of the organ or organs required by the human. The modified organ(s) then is (are) harvested and transplanted into the human patient.

As an illustrative embodiment, mesenchymal stem cells from a pediatric patient with a severe congenital heart defect are administered to a pig fetus. After birth, the pig would be raised to an appropriate age and/or size such that its heart approximates the size of the patient's heart. The modified heart then would be removed and transplanted into the pediatric patient.

The above description of the invention and the following examples are by way of illustration only. Other permutations and practices of the invention will be readily envisioned by one of ordinary skill in the art by view of the above in conjunction with the appended drawings. Therefore, such permutations and variations are within the scope of the present invention.

#### EXAMPLE 1

Human bone marrow aspirates routinely used for the isolation of the mesenchymal stem cells (MSCs), were purchased from Poietic Technologies, Gaithersburg, MD. Fresh bone marrow was obtained by routine iliac crest aspiration from normal human donors after informed consent was obtained. MSCs were isolated as previously described. (Pittenger, et al., Science, Vol. 284, pgs. 143-147 (1999)). In brief, 10mL of bone marrow aspirate was added to 20mL of Control Media, Dulbeccos Modified Essential Media (Gibco/BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Hyclone Inc., Logan, UT) from selected lots, and centrifuged to pellet the cells and remove the fat. The cell pellet was resuspended in Control Media and fractionated on a density gradient generated by centrifuging a 70% Percoll solution (supplier) at 13,000g for 20 minutes. The MSC enriched low density fraction was collected, rinsed with Control Media, and plated at  $1 \times 10^7$  nucleated cells/60 mm<sup>2</sup> dish. The MSCs were cultured in Control Media at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Upon reaching near-confluence, the cells were detached with 0.25% trypsin containing 1mM EDTA (Gibco/BRL) for 5 minutes at 37°C. The cells were washed with Control Media and resuspended at  $5 \times 10^6$  MSCs/mL in Control Media containing 5% DMSO (Sigma Chemical Co., St. Louis, MO). The cells were then stored in liquid nitrogen for use in the subsequent experiments.

To determine the ability of human mesenchymal stem cells (MSC's) to home to and engraft in hematopoietic sites and other tissues in the fetal sheep recipient, MSC's were transplanted by intravenous or intraperitoneal injection into fetal sheep:  $5 \times 10^6$  MSCs/fetus at 65 days gestation and  $50 \times 10^6$  MSCs/fetus at 85 days gestation (representing different ontologic stages of hematopoietic development in the sheep). At 50 days hematopoiesis is exclusively found in the fetal liver with no significant stroma detectable in the bone marrow. At 65 days the fetal liver remains the primary hematopoietic organ but stromal elements can be seen in the marrow with a few hematopoietic cells. At 80 days both fetal liver and bone marrow hematopoiesis are both active. In addition, the day 65 fetus does not mount an immune response to non-self hematopoietic cells, whereas the day 85 fetus is immune competent and rejects hematopoietic cell transplants routinely.

Recipients were sacrificed at 7 or 14 days after transplantation and the liver, spleen, bone marrow, thymus, lung, brain and blood were analyzed by PCR for human specific  $\beta$ -2 microglobulin. Tissues positive for human sequence were confirmed by immunohistochemistry for morphologic assessment by staining for human  $\beta$ -2 microglobulin with secondary staining with horseradish peroxidase for visualization.

## Methods

### Fetal Lamb Injections:

Time dated pregnant ewes were sedated using ketamine and mask inhalational halothane. The animal was secured on the operating table in the supine position, endotracheally intubated, and mechanically ventilated. Anesthesia throughout the procedure was maintained with a halothane/oxygen mixture titrated for adequate depth of sedation. An IV was started in the external jugular vein for infusion of lactated Ringer's solution and preoperative antibiotics. The ewe's abdomen was sterilely prepared and draped. Using standard aseptic technique, a vertical midline laparotomy was performed to allow exposure of the uterus. Fetal lambs at 65 or 85 days gestational age underwent transuterine-intraperitoneal or intravascular injection of  $5 \times 10^6$ ,  $50 \times 10^6$  MSCs/fetus, respectively. For the group undergoing intravascular injection, the ewes underwent horizontal hysterotomy with



the use of electrocautery and Babcock clamps to control bleeding at the cut uterus margin. The hind limbs and base of the umbilical cord were exteriorized and intravascular injection performed. The fetal hind limbs and umbilical cord were returned to the amniotic cavity. Amniotic fluid volume was restored with warm, sterile lactated Ringer's solution and the hysterotomy closed with a TA-90 stapler.

The maternal abdomen was closed in layers and dressed with colloidin. The ewe was placed in a movable cage where she was monitored until completely recovered from anesthesia. The ewes were continuously monitored until completely alert, and were able to stand, eat and drink. At this time, the animal was transported back to her holding room. Buprenorphine was administered to alleviate postoperative pain. The animals were checked by the investigator's team 4-8 hours later, and then once or twice daily. Further doses of buprenorphine were administered every 8 hours as needed for pain. Also, antibiotic (liquamycin) was given daily for 5 days. On each postoperative visit: the general well-being of the animal was assessed; the wound examined both visually and by palpation to detect signs of infection or dehiscence; and the vagina was examined externally to look for a discharge or mucous plug, both signs of preterm labor.

Animals, including ewes and lambs, were euthanized at 7 or 14 days after transplantation via initial sedation using ketamine and then lethal, intracardiac KCl injection. Various tissues, including liver, lung, bone marrow, thymus, spleen, brain, and blood were harvested for histopathology and for analysis of the presence of human engraftment. For PCR analysis of tissues (cardiac muscle, thymus, bone marrow, muscle and spleen), animals were sacrificed at 9 weeks (4 animals 65 day, 4 animals 85 day) (Figures 1-4).

#### Tissue Processing:

Fetal tissues were fixed overnight in 10% neutral buffered formalin at 4°C and paraffin embedded. For isolation of total cellular DNA, samples from each tissue were snap frozen in liquid nitrogen, and stored at -80°C for later DNA extraction.

#### Immunohistochemistry:

Serial 5µm sections were obtained from each of the paraffin-embedded tissues using a 30/50 microtome. Sections were deparaffinated, dehydrated, and rehydrated and then subjected to microwave antigen retrieval. Sections were then stained immunohistochemically for human class I antigen and SH2/SH3 antigen.

5 The latter two antigens are found on MSCs. (See U.S. Patent No. 5,837,539.)

#### Polymerase Chain Reaction:

Total cellular DNA from the organs mentioned above were isolated using DNazol. Specific primers for human class I antigen were selected based on the published human class I sequence. In brief, 1µg DNA were added to each 0.65 mL microcentrifuge tube and placed on ice. A master mix was prepared and added on ice such that the final concentration of reagents for each sample was 2.5U Amplitaq Gold DNA polymerase (Perkin Elmer, Norwalk, CT). 200 µM deoxytriphosphates (dNTP's, Pharmacia, Piscataway, NJ), 50mM KCl, 10mM Tris-C1 (pH 8.3 at 22°C),

10 1.5mM MgCl<sub>2</sub>, 0.01% gelatin, and 1µM upstream and downstream primers. The samples were kept on ice until the thermocycler block reached 94°C, when the samples were immediately placed into the block for 9 minutes. Samples were amplified for 40 cycles of 30 seconds at 94°C followed by 30 seconds of primer annealing followed by 1 minute of extension at 72°C. Upon completing the final

15 cycle, samples were incubated for 5 minutes at 72°C. PCR products were subjected to electrophoresis through a 2.5% NuSieve/1% Seakem agarose gel containing 0.5µg ethidium bromide/mL in 1X Tris acetate running buffer. The gels were illuminated with UV 280-nm light and photographed with type 55 positive/negative Polaroid film. The negative was scanned transmissively and the intensities of the bands

20 determined using the Intelligent Quantifier densitometer. Band intensities was compared to standard curves generated with known concentrations of human DNA.

25

#### EXAMPLE 2

30 **Animals:** Time dated pregnant Western Cross sheep carrying twin gestations (Thomas Morris, Reisterstown, MD) were housed in the ALAAC approved large animal facility at the Children's Hospital of Philadelphia and fed standard chow and water ad libitum. To determine the distribution and potential for differentiation of

human mesenchymal stem cells (MSC) following *in utero* injection;  $1-2 \times 10^6$  human MSCs/kg estimated fetal weight were injected into the peritoneal cavity of 65 or 85 day gestation fetal sheep. To determine the distribution of human MSCs following *in utero* injection, the fetal sheep were sacrificed at 1 or 2 weeks or 2 or 5 months after injection and the liver, spleen, lung, bone marrow, thymus, brain, heart, skeletal muscle, cartilage, and blood were harvested and analyzed for the presence of human cells. In a small subset of 65 day gestation fetal sheep, the fetal tails were docked at the time of MSC injection and the tail wounds harvested at 1 week or 2 months after wounding for DNA isolation and immunohistochemistry.

10

**Tissue Processing:** Fetal tissue harvested above were either fixed overnight in 10% neutral buffered formalin (Fisher Scientific, Atlanta, GA) at 4°C. Bone marrow samples were then decalcified in Cal-EX (Fisher) for 12 hours, followed by a 3-4 hour wash with distilled water. Samples were then paraffin embedded as previously described (Culling, Handbook of Histopathological and Histochemical Techniques, Butterworth Co., London (1974)). In addition, samples from each tissue were snap frozen in liquid nitrogen, and stored at -80°C for subsequent total cellular DNA extraction.

15

**DNA Isolation:** Total cellular DNA from the organs mentioned above was isolated using DNAzol (Molecular Resource Center, Inc., Cincinnati, OH). In brief, approximately 100 mg of tissue was homogenized in 1mL of DNA zol. The DNA was precipitated with 0.5mL of 100% ethanol. The DNA precipitate was pelleted by centrifugation and then washed twice with 95% ethanol. The DNA pellet was then dissolved in sterile water.

25

**PCR Analysis:** To screen the ovine tissues for the presence of human cells, total cellular DNA was subjected to PCR analysis for human specific  $\beta$ -2 microglobulin using a modification of previously described methods (Gilliland, et al., Proc. Nat. Acad. Sci., Vol. 87, pgs. 2725-2729 (1990)). In brief, 1 $\mu$ g of total cellular DNA isolated from the above-mentioned tissues was added to individual 0.65mL microcentrifuge tubes and placed on ice. A master mix was prepared and added on ice such that the final concentration of reagents for each sample was 2.5U Amplitaq Gold DNA polymerase (Perkin Elmer, Norwalk, CT), 200 $\mu$ M deoxytriphosphates (dNTP's, Pharmacia, Piscataway, NJ), 50mM KCl, 10mM Tris-C1 (pH 8.3 at 22°C), 1.5mM MgCl<sub>2</sub>, 0.01% gelatin, and 1 $\mu$ M upstream and downstream primers. Specific primers for human  $\beta$ -2 microglobulin were selected based on the published human sequence (D), (upstream primer 5'-GTGTCTGGGTTTCATCAATC, downstream primer 5'-GGCAGGCATACTCATCTTTT) and shown to amplify specifically human, not ovine, DNA. The samples were kept on ice until the thermocycler block reached 95°C, when the samples were placed immediately into the block for 9 minutes. Samples were amplified for 50 cycles of 30 seconds at 94°C followed by 30 seconds of primer annealing at 55°C followed by 1 minute of extension at 72°C. Upon completing the final cycle, samples were incubated for 5 minutes at 72°C. PCR products were subjected to electrophoresis through a 2.5% NuSieve/1% Seakern agarose gel containing 0.5 $\mu$ g ethidium bromide/mL in 1X Tris acetate running buffer. The gels were illuminated with UV 280-nm light and photographed with type 55 positive/negative Polaroid film.

**Immunohistochemistry:** To verify the PCR results, immunohistochemistry for human  $\beta$ -2 microglobulin was performed as previously described. To assess differentiation, immunohistochemical staining was also performed for human CD74, a marker of thymic epithelium (F), human CD23, a marker for bone marrow stroma (G), smooth endoplasmic reticulum calcium ATPase-2 (Serca-2), a marker for cardiac, skeletal, and smooth muscle (H), or glial fibrillary acid protein (GFAP), a marker for central nervous tissue (I). In brief, paraffin sections (4-5 $\mu$ m) were collected on Superfrost Plus slides (Fisher) from each of the paraffin embedded tissues. Slides were incubated for 24 hrs. at 55°C and then deparaffinated by 30 min. immersion in xylene followed by rehydration through a graded alcohol series to deionized water over 10 min. and allowed to air dry completely. To enhance antigen retrieval, the slides were immersed in Tissue Unmasking Fluid (Ted Pella, Redding, CA). Blocking for 30 minutes at room temperature (RT) was performed using non-immune serum from the species in which the primary antibody was raised (1:20 dilution), followed by a 12 hour incubation with the specific primary antibody. The primary antibody dilutions used were as follows: human  $\beta$ -2 microglobulin (Pharmingen International, San Diego, CA, 1:200); human CD74 (Pharmingen, 1:10); or human CD23 (Vector Laboratory, Burlingame, CA, 1:10). The slides were then washed with PBS followed by a second blocking step with methanol containing 0.3% hydrogen peroxide for 30 minutes at room temperature. Slides were then rinsed with deionized water, then PBS, followed by incubation with biotinylated secondary antibody (1:200 dilution) for 30 min. at RT. The slides were washed with PBS and avidin-biotin complex added for 45 min. at RT. The slides were then rinsed well in PBS, developed with the chromagen 3,3'-diaminobenzidine. For sections stained for human  $\beta$ -2 microglobulin, CD74, and CD23 the slides then were

lightly counterstained with hematoxylin. For cardiac muscle and brain, the human  $\beta$ -2 microglobulin was developed first using nickel chloride as the chromagen, and then subjected to a secondary immunohistochemical staining for SERCA-2 (Vector Laboratory, 1:50 dilution) or GFAP (Vector Laboratory, 1:100 dilution),  
5 respectively, as previously described (Van Der Loos, et al., Histochem. J., Vol. 25, pgs. 1-11 (1993); VanDer Loos, et al., J. Hist. Cyto., Vol. 42, pgs. 289-294 (1994)). Secondary staining was developed using Vector VIP substrate kit (Vector Laboratory). No counterstaining was performed on these double-stained slides.

10

## Results

### PCR Assessment of Human MSC Distribution

In order to assess the early distribution of human MSCs following *in utero* transplantation, PCR for human specific  $\beta$ -2 microglobulin DNA sequences was  
15 performed on DNA isolated from liver, spleen, lung, bone marrow, thymus, brain, heart, skeletal muscle, and blood from fetuses transplanted at either 65 or 85 days gestation. Tissue was harvested at 2 weeks, 2 months, or 5 months after *in utero* transplantation. Two weeks after transplantation human  $\beta$ -2 microglobulin DNA was detected in all tissues examined in fetal sheep transplanted at 65 and 85 days  
20 gestation (Figure 6A and Table 1), with the exception of skeletal muscle. Cartilage was not examined. After 2 months, human DNA was still detected in all tissues examined from fetuses transplanted at 65 days gestation including cartilage, with the exception of brain. In fetuses transplanted at 85 days gestation, human DNA was detected after 2 months in the spleen, bone marrow, thymus, heart, and blood. Five  
25 months after *in utero* transplantation (3 months after birth), human DNA was

detected in the bone marrow, thymus, spleen, lung, cartilage, and blood of fetuses transplanted at 65 days and in the heart, brain, skeletal muscle, and blood of fetuses transplanted at 85 days. Although the pattern of human cell distribution in individual animals differed, human specific sequences were detected in all animals  
5 transplanted at the time of sacrifice (Table 1).

#### **Immunohistochemical Assessment of Human MSC Distribution**

The presence of human cells in tissues positive by PCR was confirmed by immunohistochemistry, using an antibody specific for human  $\beta$ -2 microglobulin  
10 (Pharmingen, San Diego, CA, mouse IgM, Clone TU99) a component of the Class I antigen complex. Negative controls, consisting of tissues from transplanted sheep that were negative by PCR, and of matched tissues from normal sheep, confirmed the human specificity of the staining (data not shown). Many human MSCs were seen in pre- and post-natal hematopoietic and lymphopoietic tissues including the  
15 fetal liver, bone marrow, spleen, and thymus (Figures 6B-6E). Multiple human MSCs could often be appreciated in a single high-power field (Figures 6B and 6C) in these tissues. Human cells were also identified in non-lymphohematopoietic sites including the heart, skeletal muscle, cartilage, perivascular areas of the CNS, and lung (Figure 6F). Five months after transplantation, human cells continued to be  
20 present in multiple tissues including the bone marrow, thymus, cartilage, heart, skeletal muscle, and brain.

#### **Immunohistochemical Assessment of Human MSC Differentiation**

Differentiation of human MSCs in various tissues following transplantation  
25 was assessed by one of three techniques: 1) characteristic morphology on anti-

human  $\beta$ -2 microglobulin staining; 2) immunohistochemical double-staining for anti-human  $\beta$ -2 microglobulin and a second non-human specific differentiation marker; or 3) when available, positive staining with human specific differentiation markers proven to not cross-react with sheep cells. Using these techniques, site-specific differentiation was confirmed for human cardiomyocytes, chondrocytes, bone marrow stromal cells, thymic stromal cells, and skeletal myocytes. Human cells were identified in the CNS.

#### Cardiomyocyte Differentiation

10 To assess differentiation of human MSCs found in cardiac muscle, double-staining immunohistochemistry was performed using anti-human  $\beta$ -2 microglobulin and anti-SERCA-2 (Smooth Endoplasmic Reticulum ATPase). At 2 and 5 months after *in utero* transplantation, human cells were detected in the cardiac muscle of fetuses transplanted at 65 and 85 days gestation. These cells had similar  
15 morphology to the surrounding ovine cardiomyocytes and also double-stained with human  $\beta$ -2 microglobulin and SERCA-2, consistent with human cardiomyocyte differentiation (Figures 7B and 7C).

#### Chondrocyte Differentiation

20 Chondrocyte differentiation was identified by the finding of human  $\beta$ -2 microglobulin positive cells in cartilage lacunae of lambs transplanted at 65 days and harvested at 2 months or 5 months after transplantation. Immunohistochemistry was performed using a nickel chloride-based developing technique giving the particulate appearance observed (Figures 8A and 8B). The immunohistochemical  
25 identification of human cells within the lacunae of cartilage specimens that were



DNA PCR positive for human  $\beta$ -2 microglobulin sequences represents clear evidence of human chondrocyte differentiation.

#### **Bone Marrow Stromal Differentiation**

5           To assess differentiation of human MSCs found in the bone marrow immunohistochemistry was performed using a human specific anti-CD23 antibody (Pharmingen, San Diego, CA, mouse IgG<sub>1</sub>, Clone M-L233). CD23 is the low affinity IgE receptor and has been shown to be expressed on a variety of cell types including bone marrow stromal cells (Huang, et al., Blood, Vol. 85, pgs. 3704-3712  
10 (1995); Fourcade, et al., European Cytokine Network, Vol. 3, pgs. 539-543 (1992)). At 5 months after *in utero* transplantation many human cells were seen in the marrow and were demonstrated to express CD23 (Figures 9B through 9D). These human CD23 positive cells appeared to be large cells clustered in areas with ovine hematopoietic elements, consistent with bone marrow stroma.

15

#### **Thymic Stromal Differentiation**

          To assess differentiation of human MSCs found in the thymus, immunohistochemistry was performed using a human specific anti-CD74 antibody (Pharmingen, San Diego, CA, mouse IgG<sub>1</sub>, Clone LN2). At 5 months after *in utero*  
20 transplantation, multiple human cells were detected in the thymus that strongly expressed CD74 (Figures 10B through 10D), an MHC associated invariant chain expressed on thymic stromal cells (Schlossman, et al., Leukocyte Typing V: White Cell Differentiation Antigens, Oxford University Press, New York (1995)). These cells were large and were similar in morphologic appearance to nearby ovine thymic  
25 epithelium.

### Human Cell Persistence in the CNS

To assess differentiation of human MSCs found in the brain, double-staining immunohistochemistry was performed using anti-human  $\beta$ -2 microglobulin and anti-  
5 GFAP (Glial Fibrillary Acid Protein). At 5 months after *in utero* transplantation, numerous  $\beta$ -2 microglobulin positive human cells were detected on the surface of the brain in the perivascular areas within the giral sulci (Figure 11). The differentiation state of the cells was not determined.

### 10 Assessment of Human MSC Participation In Tissue Repair after Wounding:

To assess the possible participation of human MSCs in tissue repair after wounding, tail wounds were created in five 65 day gestation fetal sheep at the time of MSC injection. One animal was sacrificed at one week and four animals at 2  
15 months. Human  $\beta$ -2 microglobulin DNA was detected by PCR in the one tail wound at 1 week and in one of four tail wounds at 2 months. The PCR results were verified by human  $\beta$ -2 microglobulin immunihistochemistry (data not shown). The cells expressing human  $\beta$ -2 microglobulin in the tail wound appeared in the dermis and dermal appendages and had the morphologic appearance of fibroblasts consistent with participation in the wound healing response.

20

### Discussion

Mesenchymal stem cells are of increasing interest to the emerging fields of tissue engineering, cellular transplantation, and gene therapy because of their availability in bone marrow, their relative ease of expansion in culture, their  
25 amenability to genetic manipulation, and most importantly, their capacity for

differentiation into multiple mesenchymal tissues. These properties support potential clinical applications of: 1) large scale tissue engineering particularly for repair of musculoskeletal injury; 2) cellular therapy for diseases of mesenchymal origin such as muscular dystrophy, osteoporosis, osteogenesis imperfecta, and collagen disorders; 3) bone marrow conditioning to facilitate engraftment of autologous or allogeneic hematopoietic stem cells; and 4) gene therapy. Prenatal MSC transplantation may provide a "reservoir" of normal stem cells to replace defective cells as they become damaged in degenerative diseases with progressive cellular and organ damage.

10

Experiments utilizing porous diffusion chambers or ceramic cubes have documented the capacity of MSCs to form fibrous tissue, cartilage, or bone *in vivo* (Kadiyala, et al., Cell Transplantation, Vol. 6, pgs. 125-134 (1997)). In addition, MSCs have been shown to improve healing of segmental bone defects and cartilage defects following direct implantation into injury sites (Wakitani, et al., J. Bone & Joint Surg. - American Volume, Vol. 76, pgs. 579-592 (1994)). Of greater relevance to this study are studies that have followed the fate of MSCs or MSC like populations following intravenous or intraperitoneal transplantation. There have been two studies in mice in which cultured mouse adherent cell populations have been transplanted and documented to persist following transplantation. In the first, cells from transgenic mice expressing a human mini-gene for collagen I were used as mesenchymal progenitor donors and the fate of the cells followed after transplantation into irradiated mice (Pereira, et al., Proc. Nat. Acad. Sci., Vol. 92, pgs. 4857-4861 (1995)). Donor cells were detected in bone marrow, spleen, bone cartilage, and lung up to 5 months later by PCR for the human mini-gene, and a PCR

25

*in situ* assay on lung indicated that the donor cells diffusely populated the parenchyma. Reverse transcription-PCR assays indicated that the marker collagen I gene was expressed in a tissue-specific manner. A second study transplanted either cultured adherent cells or whole bone marrow into irradiated mice with a phenotype of fragile bones resembling osteogenesis imperfecta caused by expression of the human minigene for type I collagen (Pereira, et al., Proc. Nat. Acad. Sci., Vol. 95, pgs. 1142-1147 (1998)). With either source of cells, a similar distribution of engraftment was documented as observed in the previous study and in addition, fluorescence *in situ* hybridization assays for the Y chromosome indicated that, after 2.5 months, donor male cells accounted for 4-19% of the fibroblasts or fibroblast-like cells obtained in primary cultures of the lung, calvaria, cartilage, long bone, tail, and skin.

Our study is the first to document directly multipotential differentiation of a relatively well characterized MSC population, *in vivo*, after transplantation.

15

The results help define specific aspects of MSC transplant biology. First, MSCs, although very large cells, can be transplanted, and are capable of homing to and engrafting in multiple tissues, even when transplanted into the fetal peritoneal cavity. This requires the transplanted MSC to cross endothelial barriers, integrate into host tissue microenvironments, and survive with available growth factors and regulatory signals. Our findings of a variable pattern of long-term MSC engraftment, following initial engraftment in nearly all tissues studied, supports a model of non-selective homing with subsequent selective long-term survival in specific tissues. This may be a function of the ability of specific microenvironments to support the engraftment and differentiation of MSCs, or alternatively, the loss of

25

engraftment from some tissues may be due to heterogeneity of the transplanted population with respect to differentiation potential or replicative capacity. Immune mediated rejection is less likely since the pattern of engraftment was not limited to immune privileged sites, and an immune mechanism should result in eradication of  
5 donor cells.

A second observation of this study is that MSCs are capable of site specific, multipotential differentiation and tissue integration following transplantation. Human MSCs have been shown *in vitro* to differentiate into adipocytic,  
10 chondrocytic, or osteocytic lineages (Pittenger, supra). Less well characterized MSC populations from other species have been induced *in vitro* toward myocytic differentiation. This study confirms *in vivo* chondrocytic differentiation and for the first time clearly demonstrates *in vivo* cardiomyocytic and myocytic differentiation of a defined human MSC population. MSCs derived from bone marrow from  
15 multiple species have been demonstrated to support hematopoiesis with equal or greater efficacy than stromal layers formed in long term Dexter cultures. Our study supports the role of MSCs in stromal support of hematopoiesis, both in the fetal liver, and postnatal bone marrow. We found multiple large human cells intimately associated with clusters of hematopoiesis in the fetal liver at 2 and 9 weeks after  
20 transplantation. In addition, large cells that stained positively for human specific CD23, were identified in the bone marrow at 9 and 22 weeks after transplantation. CD23 has been identified as a low affinity IgE receptor as well as a functional CD21 ligand (Huang, et al., 1995; Aubry, et al., Cell, Vol. 57, pgs 1073-1081 (1989)) present on a variety of hematopoietic cells as well as bone marrow stromal cells  
25 (Fourcade, et al., 1992). Our interpretation of CD23 positive cells in this study as

"stromal" is based on the large size of the cells and the well documented absence of human hematopoietic cells in either the donor cell population or the recipient bone marrow.

5 A relatively surprising finding was the presence of large thymic cells that stained positive for human specific  $\beta$ -2 microglobulin and CD74. CD74 is a cell surface MHC class II-associated invariant chain molecule that is expressed on B-cells, Langerhans cells, dendritic cells, activated T-cells, and thymic epithelium (Schlossman, et al., 1995). The morphology of CD74+ cells in this study appears  
10 similar to the ovine thymic epithelial cells in the surrounding thymus. The precursor of thymic dendritic cells is thought to be the hematopoietic stem cell, whereas the origin of the thymic epithelial cell is unknown. Our data support a mesenchymal origin for the thymic epithelial cell as a "stromal" supporting cell in the thymus. Finally, the presence of human fibroblast like cells in tail wound sites suggests that  
15 MSCs are capable of appropriate differentiation for participation in repair of damaged tissues.

The persistence of human cells observed in this xenogeneic model, even when transplanted after the development of immunocompetence in the sheep fetus is  
20 intriguing. Potential mechanisms for tolerance include failure of immune recognition, local immune suppression, or thymic deletional tolerance. Human MSCs are known to express Class I HLA antigen but do not express Class II, which may limit immune recognition. Although thymic stromal cells are known to participate in thymocyte positive and negative selection and host thymic antigen  
25 presenting cells are capable of facilitating clonal deletion of donor reactive

lymphocytes after *in utero* HSC transplantation (Kim, et al., J. Pediatr. Surg., Vol. 34, pgs. 726-730 (1999)), neither mechanism would account for tolerance after the appearance of mature lymphocytes in the peripheral circulation. *In vitro*, MSCs added to mixed lymphocyte cultures, however, have been shown to non-specifically ablate alloreactivity by an as yet unknown mechanism (Schwartz, 1989; Sha, et al., 1988; Kim, et al., 1999). It is believed that the persistence of MSCs in this model results from a combination of minimal immunogenicity, and local immune suppression.

10           It is to be understood, however, that the scope of the present invention is not to be limited to the specific embodiments described above. The invention may be practiced other than as particularly described and still be within the scope of the accompanying claims.

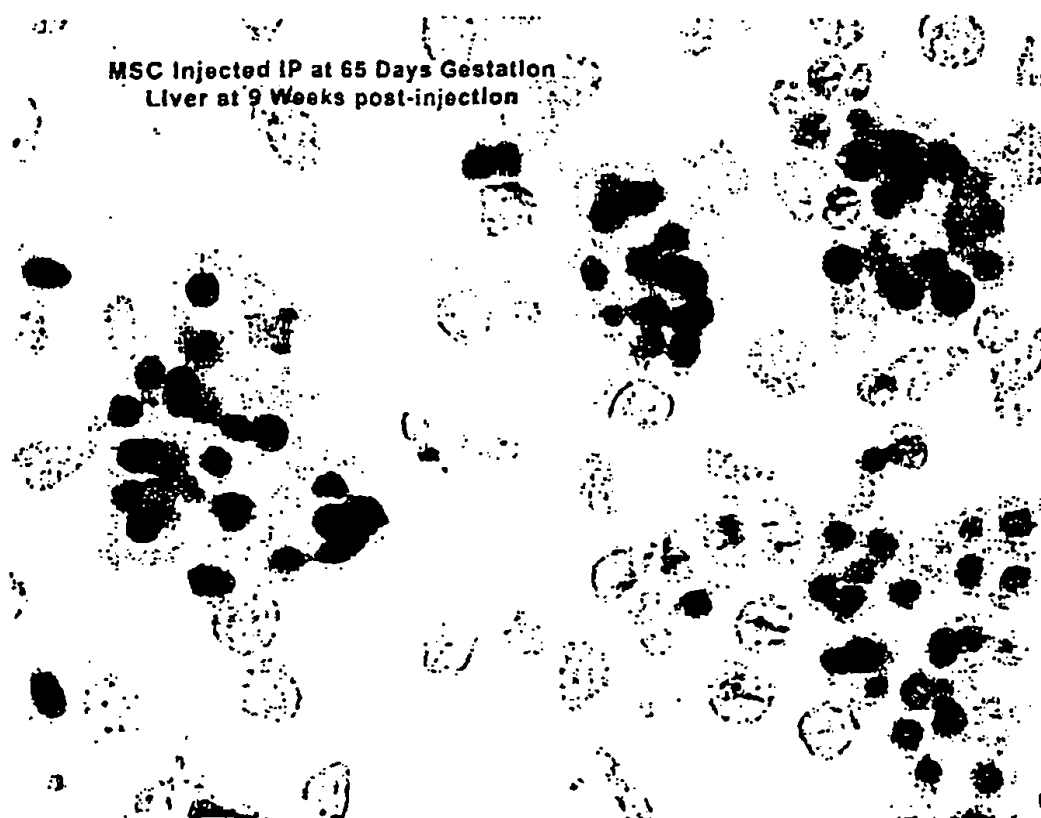
### CLAIMS

1. A method for treating a fetus comprising administering mesenchymal stem cells to the fetus.
- 5 2. The method of Claim 1 wherein the mesenchymal stem cells differentiate in vivo.
3. The method of Claim 1 wherein the mesenchymal stem cells are modified  
10 with exogenous genetic material.
4. The method of Claim 1 wherein said fetus is non-human.
5. The method of Claim 4 wherein said mesenchymal stem cells are human.
- 15 6. A method of engrafting mesenchymal stem cells, comprising: administering mesenchymal stem cells to a fetus in utero.
7. The method of Claim 6 wherein said fetus is non-human.
- 20 8. The method of Claim 7 wherein said mesenchymal stem cells are human.
9. A method of preparing an organ for transplantation, comprising:  
25 (a) administering mesenchymal stem cells to a non-human fetus in utero; and  
(b) harvesting said organ.
- 30 10. The method of Claim 9 wherein said non-human fetus is ovine.
11. The method of Claim 9 wherein said mesenchymal stem cells are human.
12. The method of Claim 9 wherein said organ is harvested after the birth of said  
35 fetus.
13. A method of xenotransplantation comprising: transplanting the organ of Claim 12 into a human patient.
- 40 14. The method of Claim 13 wherein said organ is a heart.
15. The method of Claim 13 wherein said organ is a pancreas.
16. The method of Claim 13 wherein said organ is a kidney.
- 45



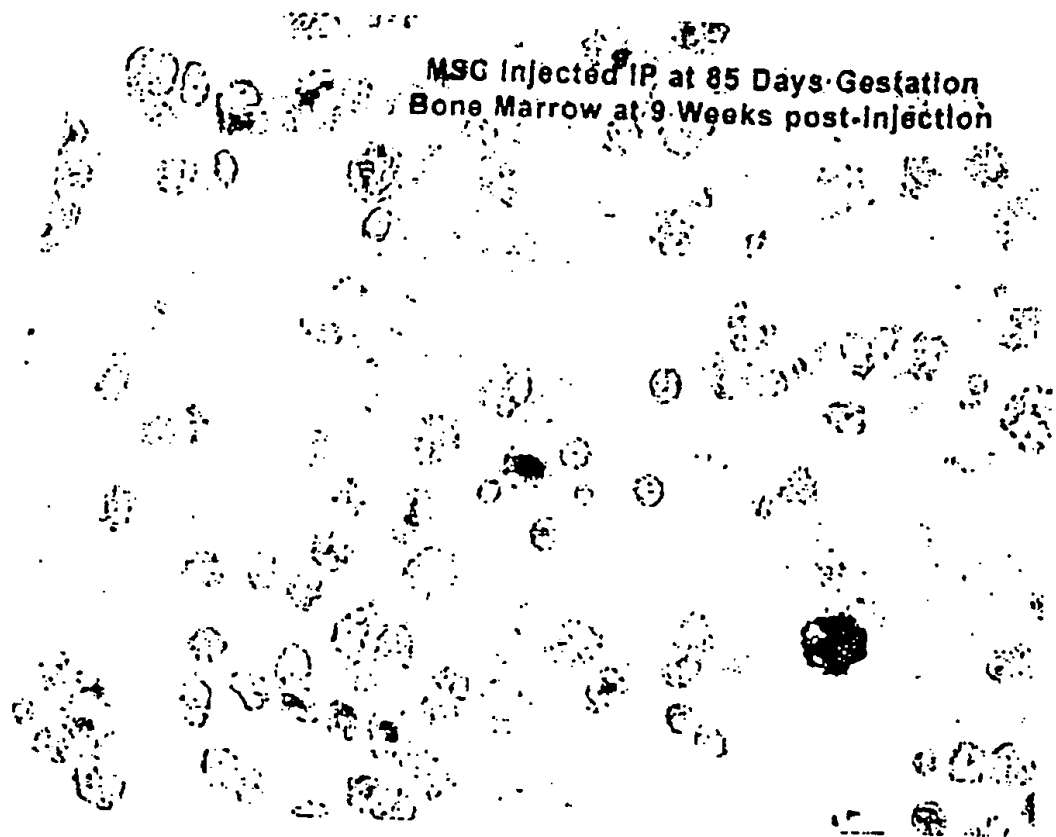
17. The method of Claim 13 wherein said organ is a liver.
18. The method of Claim 13 wherein said organ is skin.
- 5 19. The method of Claim 13 wherein said organ is a thymus.
20. The method of Claim 13 wherein said organ is a spleen.
21. The method of Claim 13 wherein said organ is bone marrow.
- 10 22. The method of Claim 13 wherein said organ is cartilage.
23. The method of Claim 13 wherein said organ is bone.
- 15 24. A hybrid organ comprising an organ of an animal of a first species and mesenchymal stem cells from a second species.
25. The hybrid organ of Claim 24 wherein the mesenchymal stem cells have differentiated into cells of that organ.
- 20 26. The hybrid organ of Claim 24 wherein said first species is non-human, and said second species is human.
- 25 27. The hybrid organ of claim 24 wherein said organ of an animal of a first species is selected from heart, lung, kidney, pancreas, skin, liver, spleen, thymus, bone, cartilage, and bone marrow.

# FIG. 1



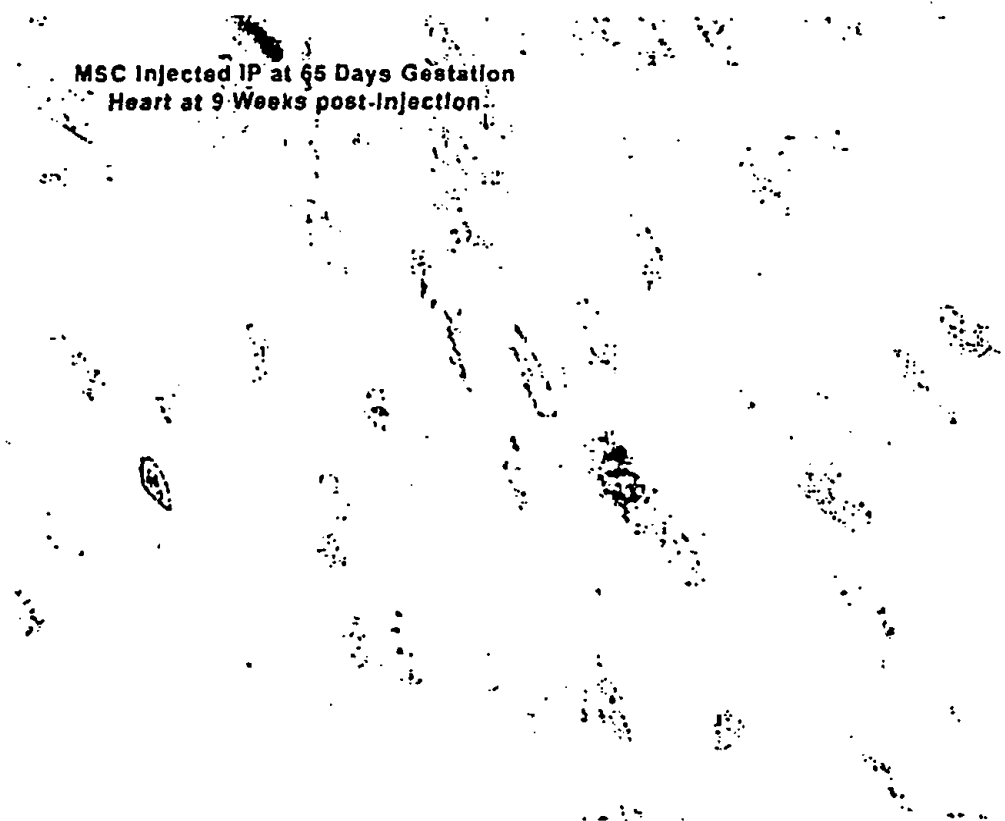
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# FIG. 2



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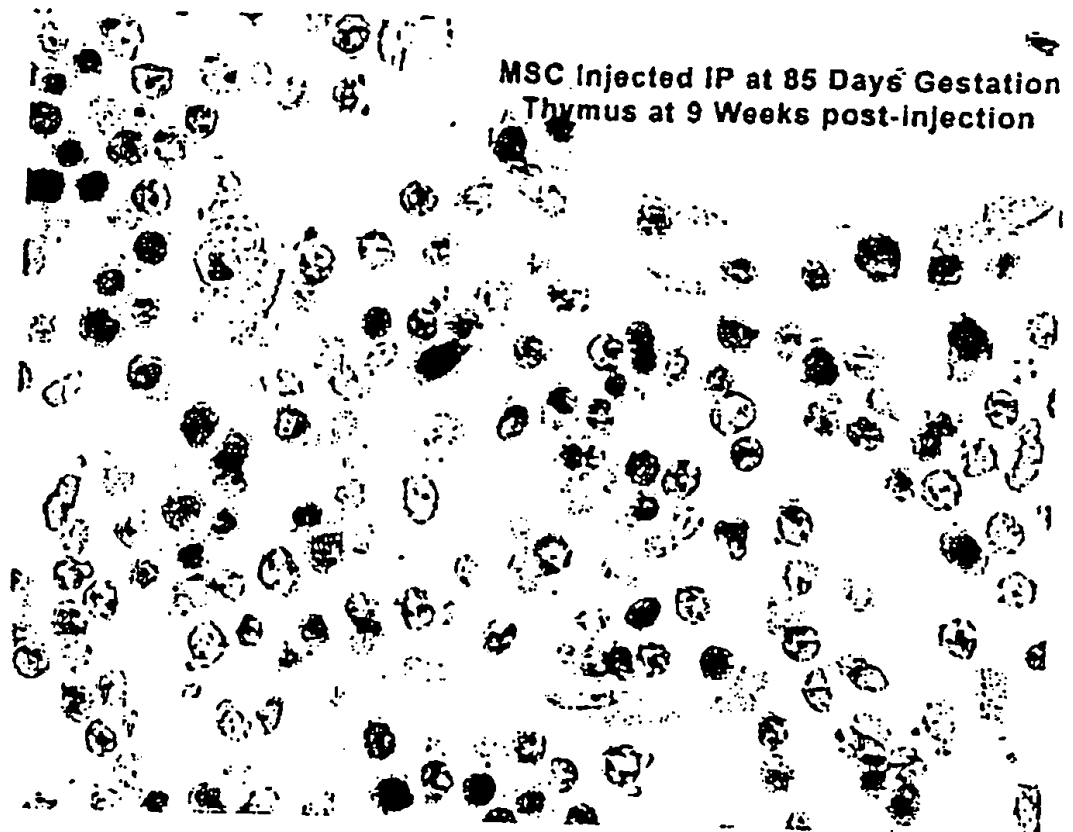
# FIG. 3



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FIG. 4



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FIG. 5

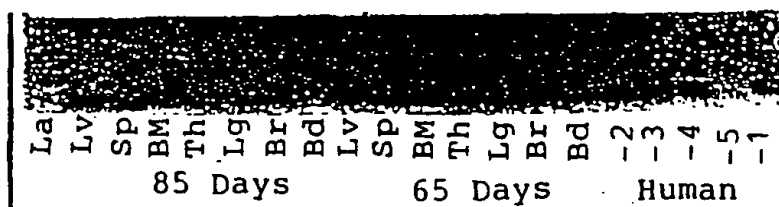


FIG. 7A

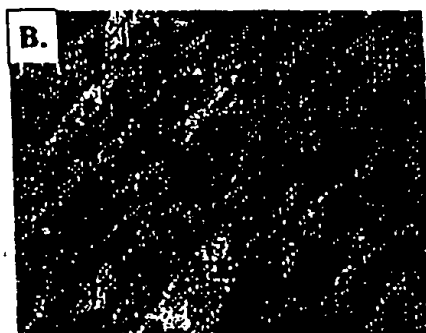


FIG. 7B



FIG. 7C

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FIG. 6A

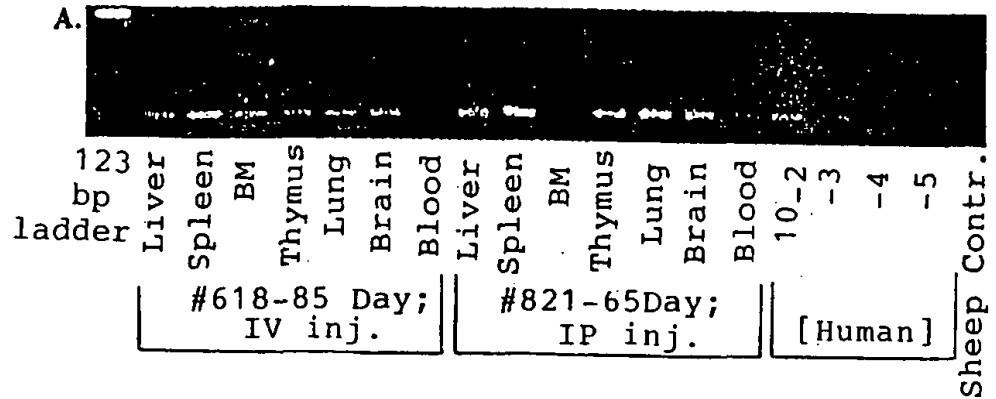


FIG. 6B

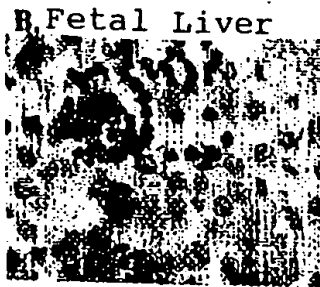


FIG. 6C

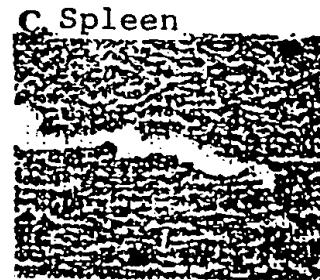


FIG. 6D

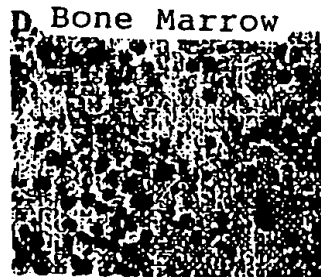


FIG. 6E

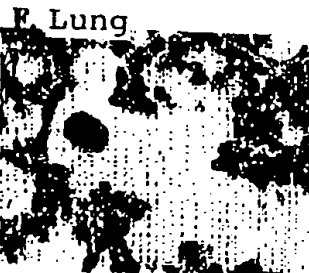
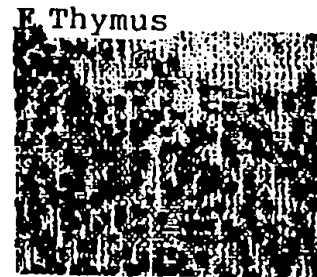


FIG. 6F

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FIG. 8A

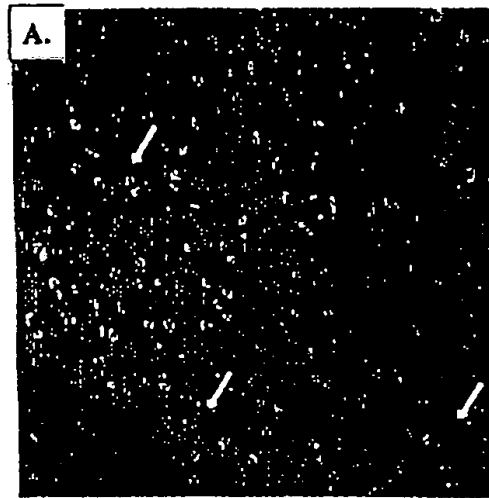
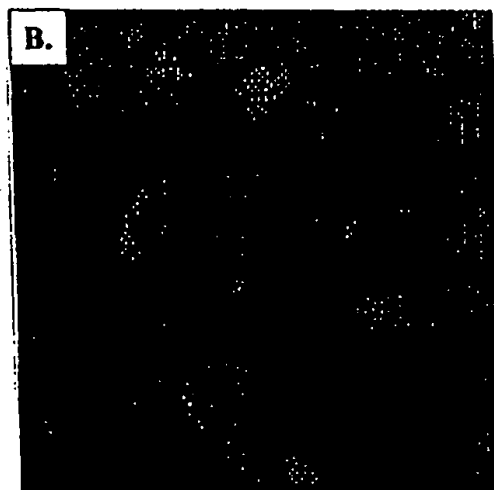


FIG. 8B



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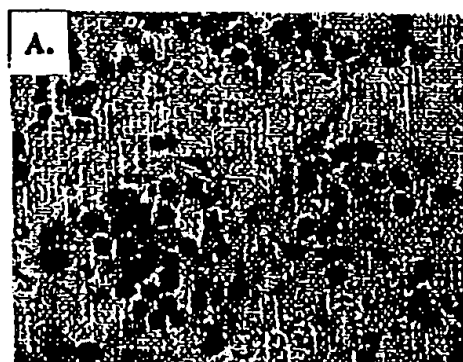


FIG. 9A

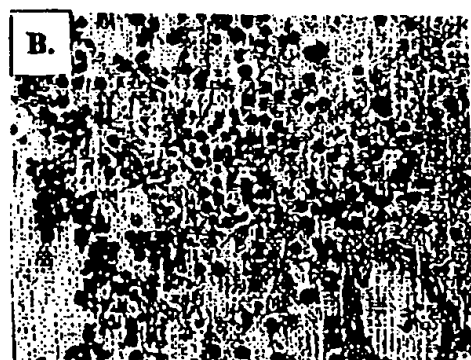


FIG. 9B

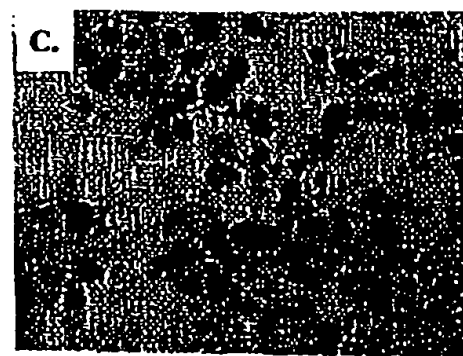


FIG. 9C

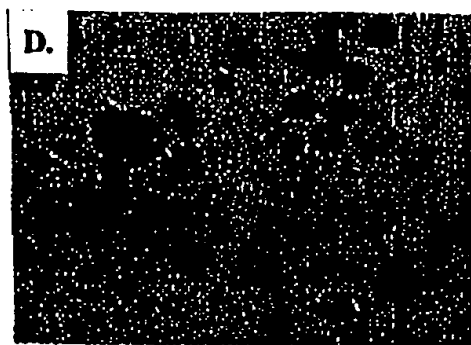


FIG. 9D

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FIG. 10A



FIG. 10B



FIG. 10C

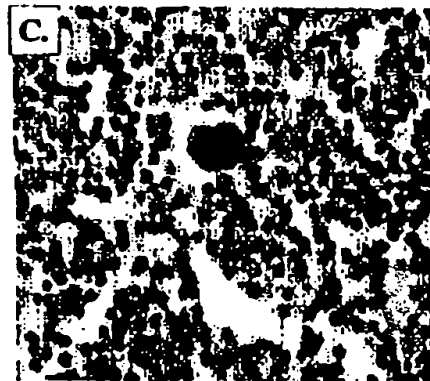
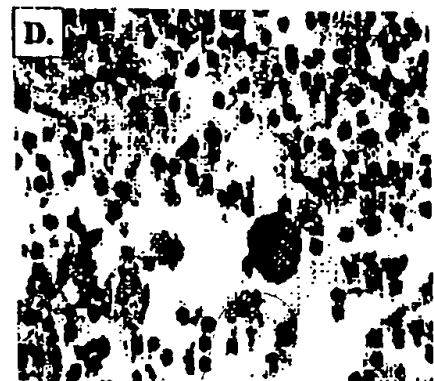


FIG. 10D



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FIG. 11A

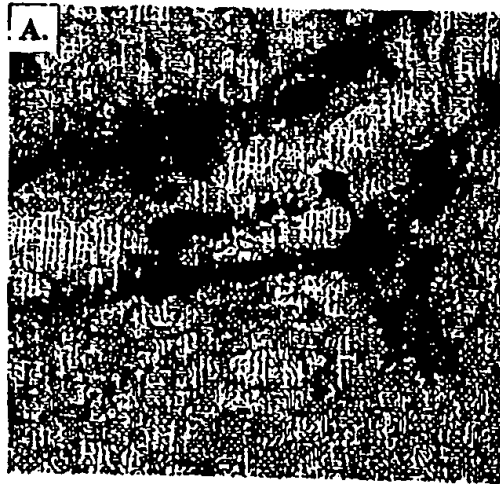
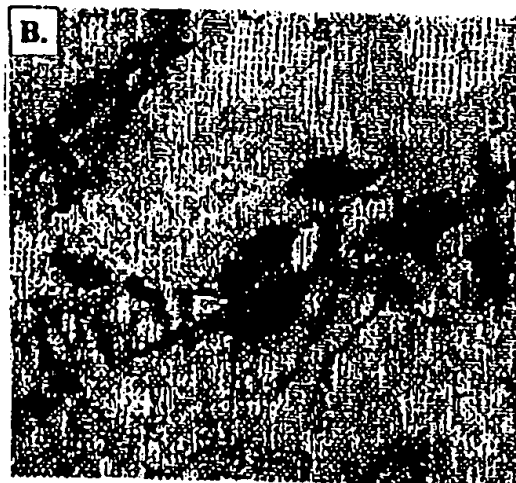


FIG. 11B



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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 7 :</b> <b>A61K 35/12, 35/28, 48/00, A01K</b> <b>67/027, C12N 5/00, 15/00</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/29002</b> <b>(43) International Publication Date:</b> 25 May 2000 (25.05.00)
<b>(21) International Application Number:</b> PCT/US99/26927 <b>(22) International Filing Date:</b> 12 November 1999 (12.11.99) <b>(30) Priority Data:</b> 60/108,357 13 November 1998 (13.11.98) US <b>(71) Applicant (for all designated States except US):</b> OSIRIS THERAPEUTICS, INC. [US/US]; 2001 Aliceanna Street, Baltimore, MD 21231-2001 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> THIEDE, Mark [US/US]; 12572 Markaire Drive, St. Louis, MO 63146 (US). FLAKE, Alan [US/US]; 1131 Springmont Circle, Bryn Mawr, PA 19010 (US). <b>(74) Agents:</b> LILLIE, Raymond et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i> <b>(88) Date of publication of the international search report:</b> 5 October 2000 (05.10.00)
<b>(54) Title:</b> <i>IN UTERO</i> TRANSPLANTATION OF HUMAN MESENCHYMAL STEM CELLS <b>(57) Abstract</b> <p>The present invention is directed to mesenchymal stem cells for transplantation <i>in utero</i>. Such mesenchymal stem cells may be employed in a method of treating a fetus, or in a method of engrafting mesenchymal stem cells, or in a method of preparing an organ for transplantation.</p>		

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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US 99/26927

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K35/12 A61K35/28 A61K48/00 A01K67/027 C12N5/00  
C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A01K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SROUR E F ET AL.: "Sustained human hematopoiesis in sheep transplanted in utero during early gestation with fractionated adult human bone marrow cells" BLOOD, vol. 79, no. 6, 15 March 1992 (1992-03-15), pages 1404-1412, XP000569644 the whole document</p> <p style="text-align: center;">--- -/--</p>	1-8

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

9 June 2000

Date of mailing of the international search report

27/06/2000

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International Application No  
PCT/US 99/26927

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	pages 73-74, "Potential uses in cell and gene therapy"	4-8, 24-27
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A	WO 94 26884 A (BIOTECHNOLOGY RES & DEV) 24 November 1994 (1994-11-24) page 1 -page 4, line 26 page 7, line 8 - line 27; claims 1,35	9-23
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P,X	WO 99 46366 A (OSIRIS THERAPEUTICS INC) 16 September 1999 (1999-09-16) page 1 -page 2, line 21 page 11, line 15 -page 13, line 28	1-3
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International Application No  
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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	<p>ALMEIDA-PORADA G ET AL: "Human bone marrow stromal cell progenitors are able to induce donor specific tolerance after in utero transplantation." BLOOD, vol. 94, no. 10 SUPPL. 1 PART 1, 15 November 1999 (1999-11-15), page 40a XP002139877 41th annual meeting of the American Society of Hematology, 3-7/12-1999 abstract 167</p>	
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Information on patent family members

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